RESEARCH PAPER | Agriculture | Volume : 4 | Issue : 7 | July 2014 | ISSN - 2249-555X

Effect of Substrate Concentration on the Activity of Cellulase Produced by *Aspergillus Flavus*

KEYWORDS: Aspergillus flavus, Cellulase, Enzyme Activity, Km value and Substrate concentration

I. F. Okonkwo

Department of Agriculture and Bioresources Engineering, Nnamdi Azikiwe University, Awka, Nigeria

ABSTRACT *The purpose of this research is to determine the optimum concentration of various substrates, including carboxymethyl cellulose (CMC), filter paper and cotton wool, for inducing the highest cellulase activity in Aspergillus flavus. Substrates for cellulase activity assays can be divided into two categories, based on their solubility in water. CMC is soluble in water while cotton linter and Whatman No. 1 filter paper are insoluble. Based on the result of the research, there were increase in cellulase activity as the substrate concentrations increased and were highest at 1 % concentration in all the substrates but the rate of increase in activity was highest in cotton wool and least in CMC. The enzyme also showed normal Michaelis Menten behavior with CMC, filter paper and cotton wool substrates. However, cotton wool is a better substrate, than filter paper and CMC. This is evident from the lower Km and higher Vmax0.7 mg/ 0.08 for cotton wool, and thus is recommended for use in industrial processes involving Aspergillus flavus cellulase system.*

Introduction

Industrial interest in cellulase is high due to its wide application in various industries such as animal feed production, starch processing, malting and brewing, grain alcohol fermentation, extraction of fruit and vegetable juices, as well as manufacture of pulp, paper and textiles (Adsul et al., 2007; Kaur et al., 2007). Enzymatic hydrolysis of cellulosic materials is achieved by a sequence of reactions with the main components of cellulase complex enzymes, which include FPase, CMCase and β-glucosidase. The characteristics of all these three components in the cellulase complex are the main factors that influence the application of enzyme-based bioconversion technology. Therefore, research has been directed to discover new microorganisms that have capability to produce cellulolytic enzymes with high specific activity. Among the cellulolytic fungi, Trichoderma spp. and Aspergillus spp. have been widely studied for their ability to secrete high levels of cellulose-degrading enzymes (Zhou et al., 2008). Aspergillus spp. are the major agents of decomposition and decay and as such produce a broad range of enzymes, including cellulase. Cellulase characteristics and production by Aspergillus spp. have been well documented in the literature (Lockington et al., 2002; Ong et al., 2004; Wang et al., 2006). However, only a few reports are available on the production of cellulase by Aspergillus flavus (Ojumu et al., 2003; Solomon et al., 1990, 1999), and in many cases, have not been studied in depth. *Aspergillus flavus* is a mold of the fungal class Imperfecti (Djambatan *et al.,* 1984), scattered everywhere on a variety of substrates, among others, present in fruits, vegetables and other rotten foods and wood substances. These fungi play a role in the decomposition of polysaccharides in the wood, has a growth temperature of 30 °C – 37 °C, pH 4-6 and aerobic in nature.

The use of expensive substrate is one of the main problems in cellulase production by fermentation. Reduction of the cost of the substrate may be possible by the use of cellulosic materials that have the ability to produce high activity of cellulase (Kotchoni and Shonukan, 2002). Reduction in the production cost and improvement in cellulase yield could also be achieved using appropriate and low cost carbon and nitrogen sources in the formulation of fermentation medium (Beg et al., 2000; Senthilkumar et al., 2005).

Materials and Methods

Materials

The materials used in this research include reagents, salts, solvents, resins, and substrate media among others. Most of the materials were kindly provided by Professor F. J. C. Odibo of Department of Applied Microbiology and Brewing, Nnamdi Azikiwe University, Awka, Anambra State, Nigeria, while others were either obtained from the Research Laboratory or purchased from FinLab. Ltd., Enugu.

Methods

Isolation of Cellulolytic Fungi

Samples of rotten wood and compost were collected from Botanical garden of Nnamdi Azikiwe University, Awka, Anambra State, Nigeria. These were grown on Czapex dox medium to which 1 % carboxyl methylcellulose (CMC) was incorporated and incubated for 48 h at room temperature.

A total of seven dominant colonies were isolated and purified by successive subculture on fresh Czapex dox medium. The cellulolytic activities of the colonies were determined by point inoculation of each fungal isolates on Czapex dox-carboxy methyl cellulose medium and incubating for three days. After the incubation, zones of clearing as an indication of cellulolysis is detected by flooding the cultures with 0.5 % congo-red for 15 min. and distaining with 1 M sodium chloride for 10 min. (Okonkwo and Odibo, 2013). The zones were measured on a scale and result recorded.

Identification of the Fungal Species

The colony with the highest zone diameter was observed by slide culture technique under the microscope with the methylene cotton blue stain, for the characteristic morphological features. The manual of soil fungi and colour plate was used for the identification.

Screening for Cellulase Production

The selected isolates were cultivated in a Mandel and Weber (1969) medium containing $(NH_4)_2SO_4$ 1.4 g, KH_2PO_4 2.0 g, Urea 0.3 g, $MgSO_4 \times 7H_2O$ 0.3 g, $CaCl_2$ 0.3 g, $FeSO_4 \times 7H_2O$ 0.005 g, $ZnSO_4 \times 7H_2O$ 0.0014 g, $MnSO_4 \times H_2O$ 0.0016 g, $CoCl_2$ 0.002 g, Tween-80 2.0 ml, CMC 10.0 g, distilled water 1 L and pH 6.8. A loopful of conidia was inoculated into 100 ml of the sterilized medium in a 500 ml flask and incubated at 35 °C on a Stuart orbital shaker model S150 for 7 days at 200 rpm. After the incubation, the broth culture was subjected to centrifugation at 4000 rpm for 20 min using Centurion Centrifuge to remove the mycelia and other insoluble materials. The supernatant was recovered and used for the enzyme assays.

Enzyme Purification

Purification of the cellulolytic enzyme using ion exchange gave rise to cellulase yield of 3.8 % with purification fold of 10.5 (Table 1); further purification using Sephadex G-200 gel

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filtrations showed a homogenous preparation of the enzyme. Purification profile summaries of the processes are presented in Table 1 with purification fold of 20 and percentage yield of 4.9 and specific activity of 4 U mg^{-1} protein.

Table 1: Enzyme Purification Profile

Purification Step	Enzyme Vol. ml^{-1}	Enzyme Activity Units/ml	Protein content Mg/ml	Total Activity Units/ml	Total Protein mg/ml	Specific Activity Unit/mg protein	Purification Fold	% yield
Crude Supernatant	700.00	2.50	12.50	1750.00	8,750.00	0.200	1.00	100
Concentration	100.00	3.08	4.00	308.00	400.00	0.77	3.85	17.60
Ion-Exchange	20.00	3.36	1.60	67.2	32.0	2.10	10.50	3.80
Gel filtration Sephadex G-200	18.00	4.81	1.20	86.58	21.60	4.00	20.00	4.90

Effect of Substrate Concentration on Enzyme Activity

The effect of substrate concentration on the activity of the purified enzyme was studied using different concentrations of the various substrates (CMC, filter paper and cotton wool) ranging from 0 to 1.2 mg/ml in 0.2 M phosphate buffer (pH 6.8). The kinetic constants (K_m, V_{max}) of the data were estimated by double reciprocal plot of Line weaver and Burk as described by Lehninger (1976).

Enzyme Assays

Carboxymethyl Cellulose (CMC) Saccharifying Activity

An appropriately diluted (1:2) enzyme sample (0.5 ml) was mixed with 0.5 ml of 1 % CMC (Takao et al., 1985; Stephen et al., 2003) dissolved in 0.2 M phosphate buffer (pH 6.8) and incubated for 30 min at 40 °C in a water bath (Memmert). The reducing sugar released was estimated by 3, 5- dinitrosalicylic acid method (Miller, 1959) as follows: at the end of incubation, the enzyme reaction was stopped by adding 0.5 ml of 3, 5-dinitrosalicylic acid reagent (BDH). The mixture was placed in boiling water for 10 min, after which it was cooled, and 5 ml distilled water were added. The absorbance was then read at 540 nm using the substrate solution treated in the same way as blank to zero the spectrophotometer (JENWAY), model 6405. One unit (IU) of CMCase activity was defined as the amount of enzyme required to liberate 1 µmol reducing sugar from the appropriate substrate per min under the assay conditions.

Filter Paper Saccharifying Activity

The reaction mixture containing 0.25 ml of diluted enzyme solution, 0.5 ml of 0.2 M phosphate buffer (pH 6.8) and 25 mg of whatman No. 1 filter paper strip was incubated at 40 °C for 1 h as described by Takao et al. (1985) and Stephen et al. (2003). The reducing sugar liberated was determined by 3, 5- dinitrosalicylic acid method (Miller, 1959). One unit (1 U) of filter paper activity is defined as the amount of enzyme required to liberate 1 µmole of glucose per min.

Cotton Wool Saccharifying Activity

To a mixture of 1.0 ml of diluted enzyme and 1.0 ml of 0.2 M phosphate buffer (pH 6.8) was added 50 mg of absorbent cotton wool and incubated at 40 °C for 24 h (Takao et al., 1985; Stephen et al., 2003). The reducing sugar liberated was determined by the 3, 5- dinitrosalicylic acid method described above. One unit of cotton saccharifying activity was taken to be the amount of enzyme required to liberate 1 µmole of glucose per min.

Results and Discussion

The results of varying concentrations of the substrates on the activity of the purified enzyme are presented in Figure 1. Significant differences were observed in cellulase activity as the substrate concentrations increases ($P<0.05$), but the highest activity was found at 1 % concentration in all the substrates. However further increase in substrate concentration resulted in a corresponding decrease in enzyme activity. Substrate concentration of 1% is an ideal condition for Aspergillus flavus to produce cellulase enzyme because oxygen diffusion and enzyme adsorption on the substrate will run optimally at this concentration (Stewart and Parry, 1981). Also, the rate of increase in activity was highest ($P<0.05$) in cotton wool and least in CMC. Since CMC is soluble in water while cotton linter and Whatman No. 1 filter paper are insoluble, the enzyme may be more effective on insoluble substrates (Karlsson et al., 2001).

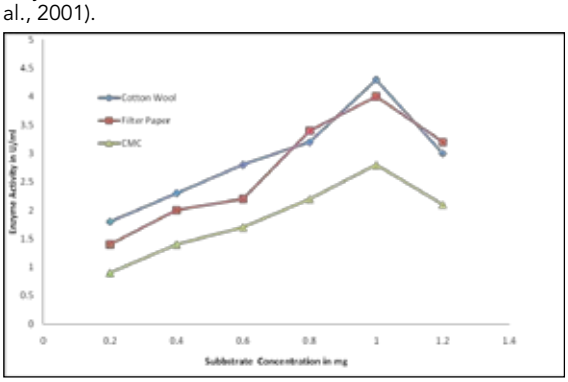

Fig. 1: Effect of Various Substrate Concentrations on the Activity of the Enzyme

Kinetic Constants

The Kinetic Constants (K_m, V_{max}) of the enzyme for the various substrates were shown in Table 2, while the comparative effect of concentration of substrates on the Mean ± SE Activity and Michelis Menten equation of the Enzyme are given in Table 3. Also the line weaver Burk plot of the linear transformation was given in Figure 2.

Table 2: The Kinetic constants (Km and Vmax) of the enzyme on various substrates

Substrate	Km	Vmax
CMC	2.20	0.32
Filter Paper	1.60	0.20
Cotton wool	0.08	0.70

Table 3: Comparative Effect of Concentration of Substrates on the Mean ± SE Activity and MichelisMenten equation of the Enzyme

Concentration	Activity		
	CMC	Filter Paper	Cotton Wool
0.2	0.90^a	1.40^b	1.80^c
0.4	1.40^a	2.00^b	2.30^c
0.6	1.70^a	2.20^b	2.80^c
0.8	2.20^a	3.40^b	3.20^b
1.0	2.80^a	4.00^b	4.30^c
1.2	2.10^a	3.20^b	3.00^b
	MichelisMenten equation (1/S)		
0.5	1.10^c	0.70^b	0.50^a
2.5	0.70^c	0.50^b	0.40^a
1.7	0.50^c	0.40^b	0.30^a

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1.3	0.40[c]	0.20[a]	0.30[b]
1.1	0.30[b]	0.25[ab]	0.20[a]
0.8	0.40[b]	0.30[a]	0.30[a]

***Means bearing different superscripts in the same row are significantly different (P<0.05).**

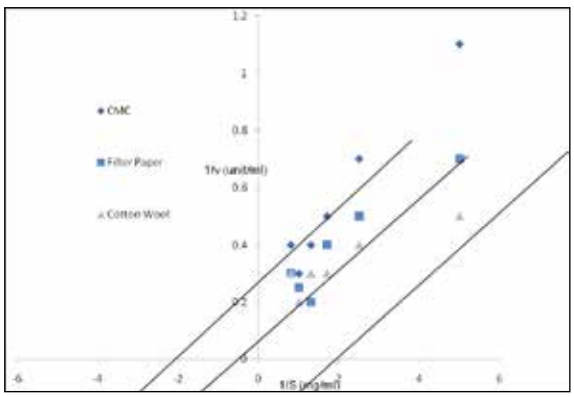

Figure 2: Line Weaver Burk Plot for Determination of Km value of Cellulase Enzyme from Aspergillus flavus

The enzyme also showed normal Michaelis Menten behavior with CMC, filter paper and cotton wool substrates. This is supported by Bakare et al. (2006) who reported that the activities of cellulases were greatly influenced by the concentration of the substrate; with fixed enzyme concentration, an increase in the concentration of the substrates results in increase in enzyme activity until a saturation point is reached beyond which enzyme activity decreases. Components in the formulation of the media for the growth and production of enzymes are an important step in designing experiments. Substrates used in fermentation processes affect enzyme activity and productivity. The existence of specific substrate concentration in the medium is needed to stimulate the growth of microorganism for metabolite secreting cells. The main nutrient for growth of microorganisms is a source of carbon, nitrogen, and minerals especially carbon.

However, cotton wool is a better substrate, than filter paper and CMC. This is evident from the lower K_m and higher V_{max} 0.7mg/ 0.08 for cotton wool and thus is recommended for use in industrial processes involving Aspergillus flavus cellulase system

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